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(54) Title: METHODS AND MEANS FOR SELECTING PEPTIDES AND PROTEINS HAVING SPECIFIC AFFINITY FOR A TARGET

(57) Abstract

    The invention provides novel methods and means for selecting peptides and for other proteinaceous molecules having specific binding affinity for a target. The binding peptides/proteins are displayed on replicable display packages, preferably phages. The binding peptides are tested for this binding affinity to the target. In one embodiment, the target is provided in the form of peptides displayed on a solid phase, in another the target is presented in a tissue section.

Title: Methods and means for selecting peptides and
proteins having specific affinity for a target.

This invention relates to the field of proteinaceous
biological molecules having specific affinity for a target.
More specifically the invention relates to such molecules
derived from specific binding peptides such as proteins such

5   as antibodies, in particular monoclonal antibodies or
specific binding derivatives or fragments thereof. In an
exemplary embodiment the invention relates to peptides and
antibodies which are more or less randomly produced as large
collections of different molecules (libraries) expressed on

10   the surface of a replicable genetic package. From these
libraries peptides or antibodies are affinity-selected for
binding to the target molecule.

Specific binding affinities between biological molecules or

15   derivatives thereof have been used throughout applied
biotechnology, molecular biology, medical biology, etc. for a
very long period of time for many different purposes. Some of
the most commonly applied biological molecules having a
specific binding affinity are antibodies. Antibodies are

20   complex proteins usually composed of two light chains and two
heavy chains which comprise constant regions and variable
regions which variable regions again comprise hypervariable
domains, which determine the binding specificity of the
antibody (complementarity determining regions, (CDR)).

25   Antibodies are part of the normal defense system of higher
vertebrates. They are produced by cells of the immune system.
Until 1975 antibodies were obtained by administrating an
antigenic substance to an animal (often a rodent) and
harvesting the antibodies produced by the animal in its

30   immune response. This resulted in mixtures of antibodies
having many different specifities for different binding sites
on the antigenic substance. These mixtures are referred to as
polyclonal antisera.

The advent of monoclonal antibodies (Kohler and Milstein
(1975) Nature 256:495) represented a significant technical
breakthrough with important consequences both scientifically
and commercially. Monoclonal antibodies are conventionally
5   made by fusing antibody-secreting plasma cells from an
immunized animal with a tumor cell line in order to
immortalize the former plasma cells. The immortalized
hybridomas are screened for the production of antibodies with
the desired specificities. Selected hybridomas are expanded
10 to large numbers and the monoclonal antibodies secreted into
the medium are harvested and purified for application.
Since the arrival of hybridoma technology, monoclonal
antibodies have found broad application in procedures aimed
at examining and identifying many antigens in many formats
15 from pregnancy tests to AIDS tests. They are also widely
applied in research laboratories for use in studying and
isolating a broad variety of molecules and for
immunohistochemistry, i.e. identifying certain cells in
tissues. For instance, monoclonal antibodies that are
20 reactive with specific antigens expressed on subpopulations
of cells can be utilized to detect these cells in tissue
sections using immunohistochemical or immunofluorescent
approaches. The inherent advantages of monoclonal compared to
polyclonal antibodies is that they react only with one
25 specific epitope and that they are standardized from batch to
batch and from laboratory to laboratory.

Despite of all the advantages obtainable using monoclonal
antibodies, there are a number of limitations associated with
30 hybridoma technology and therefore with the monoclonal
antibodies that can be obtained. For instance, the number of
hybridomas that can be established and screened for the
secretion of a certain antibody specificity is limited. The
immunization protocol and the manner in which the animals
35 immune system handles the antigen favors the selection of
hybridomas scereting antibodies against immunodominant

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epitopes, whereas other specificities will not be obtained
because the immunizing antigen or some of its epitopes are
not recognized by the animals immune system. In addition, the
MoAbs obtained from animals are hardly suitable for

5   therapeutical use in humans because they evoke an immune
respons. Some of these drawbacks may be circumvented by using
antibody engineering, aiming at chimeric antibodies of which
the variable regions are of animal origin and the constant
regions are of human origin (the constant region is

10   responsible for the bulk of the immune response). However
these chimeras still evoke an immune response. Another
possibility to avoid the immune response is a technique
called CDR-grafting, whereby only the CDR's are of animal
origin, which CDR's are inserted in a human antibody

15   framework. These humanized antibodies are rather difficult to
make and often loose some of their binding affinity.

Another way of modifying antibodies is trying to obtain the
smallest fragment that will still specifically bind the

20   antigen (or to be more precise the epitope) with a
significant affinity. The smaller the fragment, the less
immunogenic it may be. This has resulted in Fab'2, Fab
fragments, to peptide-like molecules comprising only one of
the three CDR's present on each heavy and light chain of

25   mammalian antibodies, i.e. CDR3 of the heavy chain.
In genetically engineering antibodies ways have also been
found to avoid having to express at least two different
subunits (a heavy and a light chain) for obtaining one
antibody. This has been achieved by making a fusion through a

30   peptide linker between a heavy and a light chain subunit.
This results in so-called single chain antibodies, which will
be discussed in more detail later on.

Apart from the drawbacks related to immunogenicity of

35   monoclonal antibodies, there is also a limitation in the
specificities obtainable with monoclonal antibodies. Nature

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employs a specific process in making CDR's of antibodies
through a complex process of hypermutations, rearrangements
and insertions in the gene fragment encoding the CDR,
followed by a selection of antibody producing cells in
5   primary and secondary lymphoid organs. The result of these
processes is that the variety of binding specificities that
can be produced in an animal is limited. As one result
thereof monoclonal antibodies are not always useful in the
application they are intended for, such as for example
10   immunohistochemistry.

Immunohistochemical and immunofluorescent procedures
involving tissue sections generally consist of the steps of
embedding a tissue in an embedding medium, with or withour
15   prior or subsequent fixation or freezing of the tissue,
cutting the embedded tissue in thin sections, incubating the
tissue sections with monoclonal antibodies, amplification of
the signal and visualisation of cells that have bound
antibody by an enzymatic reaction and precipitation of
20   substrate or by fluorescent labels.
A drawback of conventional monoclonal antibodies is that they
often do not bind to target epitopes in fixed tissue
sections, presumably as a result of destruction of the target
epitope by the fixation procedure. Fixation and embedding
25   procedures such as paraffin embedding an formalin fixation,
that are optimal for preservation of the anatomical structure
of the tissue and are routinely used for medical diagnosis,
lead to extensive protein denaturation thus limiting the
application of monoclonal antibodies that often bind to
30   native epitopes.
Another breakthrough in specific binding technology involves
the use of replicable genetic display packages. The term
replicable genetic display package or display package
describes a biological particle which has genetic information
35   providing the particle with the ability to replicate. The
package can display a fusion protein including an antibody,

antibo dy fragment, protein or peptide. The fusion protein is presented by the display pac kage in a context which permits the antibody to bind a target structure that is contac ted with the display package. The display package can be, for

5   example, derived from bacterial cells, bacterial spores or bacterial virusses. In phage display technology a gene fusion is made between a sequence encoding a peptide of interest and a sequence encoding a protein displayed on the outside of a phage (actually the peptide sequence is inserted in the

10   proteins encoding sequence). Libraries of very large numbers of display packages containing all kinds of different peptides within one of their surface proteins can be easily produced and selected and tested based on their binding affinity for other molecules or cells, etc.

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Parts of human antibody genes and sequences encoding single chain variable regions have been inserted in such phage genes and thus anibody fragment phage display libraries have been obtained. Display libraries of antibody variable regions may

20   be constructed by a variety of methods. In general, a large collection of antibodies or antibody-fragments is expressed on the surface of a replicable genetic display package. In an exemplary embodiment of the present invention, the display package is a phage particle which comprises an antibody-coat

25   protein fusion; the nucleotide sequences used for construction of the antibodies are of human origin. The library of surface expressed antibody molecules is brought into contact with the target antigen for affinity selection of antibodies that bind to the antigen. This procedure allows

30   the rapid screening of very large collections of displayed antibody molecules, yields human antibodies and does not require immunization of an animal. Novel specificities may be obtained from such libraries.

35   Phages expressing antibody specificities of interest are selected from libraries in a four step procedure 1) affinity

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binding of phages to the target antigen, 2) removal of
non-bound phages by washing 3) elution of bound phages and 4)
infection and propagation of eluted phages in E Coli
bacteria. A variety of targets has been successfully employed
5   to obtain phage antibodies from libraries by affinity
selection. Phage antibody affinity selections have been
performed on purified antigens coated to a solid phase, on
intact eukaryotic cells in suspension or in monolayers and on
prokaryotic cells.
10

The language "affinity binding" and "affinity selection" as
well as "differential binding" refer to the separation of
members of the peptide or antibody display library based on
the differing abilities of these molecules on the surface of
15 each of the display packages of the library to bind to the
target structure. Examples of affinity selection include
affinity chromatography, immunoprecipitation, fluorescence
activated cell sorting, agglutination and plaque lifts.

20 In all phage antibody affinity selection procedures, it is
imperative to obtain a large collection of binding phages in
the first round of selection, preferably binding to diverse
regions of the target. In subsequent rounds of selection,
procedures may be implemented to select from this collection
25 those antibodies that fulfill specific requirements.

The present invention provides further selection methods
whereby novel specificities can be easily obtained and/or
useful antibody fragments and epitopes (or even any
30 combination of two peptides or proteinaceous substances
having binding affinity for each other) can be identified and
obtained.
In one embodiment the invention provides a method for
identifying a peptide or antibody capable of specific binding
35 to a proteinaceous target, comprising displaying the peptide
or antibody on the surface of a replicable display package,

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synthesizing sets of oligopeptides derived from the
proteinaceous target on a solid phase and contacting the
specific binding peptide with the solid-phase-bound
oligopeptide to allow for binding. The peptides expressed on
5   the replicable display package may be linear or have another
conformation as for example in di-sulfide bridged circular
peptides or peptides that contain more than one disulfide
bridge and have an unprodictable conformation. In addition,
the target oligopeptides may be linear or comprise another
10   configuration.

Thus the present invention provides a method that allows the
directed pre-selection of phage antibodies (or other binding
peptides) to defined sets of oligopeptides representing
15   (sub)regions of the target molecule. For the purpose of the
present invention, the term antibody in its various
grammatical forms is art-recognized and includes
immunoglobulin molecules and immunologically active portions
and/or derivatives thereof, i.e. molecules that contain an
20   antigen binding site. The peptides are synthesized in
overlapping or non-overlapping sets of multimers on
polystyrene rods or other solid phases such as membranes or
beads. In the preferred embodiment, polystyrene rods used for
pepscan technology are used. After selection on solid
25   phase-bound rods, collections of phages enriched for
anti-oligopeptide phage antibodies can be used in subsequent
rounds to select anti-oligopeptide phages that also bind to
the target molecule in a another configuration or to select
antibodies that discriminate between various forms of the
30   target proteinaceous substance. Many examples of
anti-oligopeptide antibodies that bind to the target protein
the oligopeptide is derived from have been described in the
scientific literature. Herein oligopeptides are defined as
comprising 6-30, preferably 8-20 amino acid residues. They
35   will also be simply referred to as peptides.

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The term pepscan relates to a procedure for rapid concurrent
synthesis on solid supports of hundreds of peptides,
essentially as described (Geysen H., et al., Proc. Natl.
Acad. Sci. USA 81, 3998 (1984). In the original procedure,
5   the peptides are synthesized on polyethylene rods as solid
support. Other solid supports such a membraneous filters or
beads may also be used. Various peptide formats may be
synthesized including linear peptides and di-sulfide bridged
circular peptides.
10

Thus in one embodiment the present invention provides a
method for generating antibodies specific for sets of
peptides synthesized on a solid phase such as the
polyethylene rods used in pepscan technology. The subject
15 method generally comprises the steps of synthesizing on a
solid phase sets of peptides spanning a target molecule or a
subregion thereof; the construction of a display library of
antibodies or peptides; the selection of displayed peptides
or antibodies that bind to the solid-phage-bound peptides
20 using affinity selection and affinity absorption techniques.

In exemplary embodiments, the display library of peptides or
antibodies can be a phage display library or a display
library generated on bacterial or yeast cell surface.
25 After one or multiple rounds of selection, the binding
displayed antibodies or peptides may be subjected to
additional rounds of selection on peptides or on the target
protein in its native or denatured configuration. In
exemplary embodiments, the subject method can be used to
30 isolate anti-peptide antibodies that also bind to the target
protein in its native configuration, for example a marker
expressed on normal or malignant eukaryotic cells, or in
denatured configuration such as markers expressed on cells in
fixed tissue sections. Likewise, the subject method can be
35 used to generate antibodies which can discriminate between a
protein and other related forms of the protein, by using a

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subtractive approach. The related forms of the protein can
differ by one or more amino acids from the target protein or
differ as a result of post translational modifications such
as glycosylation.

5

In an illustrative embodiment of the subject method, a
specific antibody can be generated by affinity selection
using a synthetic antibody phage display library. The phage
library is incubated with the pepscan block containing rods
10   covered with peptides spanning the extracellular region of a
transmembrane protein and phages are allowed to bind to
individual rods. After removal of non-bound phages by
washing, bound phages are eluted from the rods and propagated
in bacterial cells. Multiple subsequent rounds of selection
15   may be performed using the same approach. Alternatively, the
binding phages from the first round of selection on solid
phase-bound peptides may be selected for binding to the
protein in another configuration such as that expressed on a
cell or a recombinant protein. In the subtractive approach,
20   phages eluted from the peptides in the first and/or
subsequent round(s) of selection may be incubated with rods
covered with other peptides, such as peptides differing one
or more amino acids from the peptide used for the initial
selection, in order to absorb phages that crossreact with
25   related molecules.
It is contemplated that the present invention can be applied
to obtain bi-specific antibodies that bind to two
non-overlapping epitopes on the same molecule of mono meric
antigen. Such antibodies have been shown to have enhanced
30   affinity due to the chelate effect (Cheong, H.S., et al.
Biochem. Biophys. Res. Comm 173, 795 (1990)). The DNA
encoding the pool of phage antibodies affinity selected for
binding to a set of overlapping peptides may be recloned into
vectors permitting the display of various formats of
35   bi-specific antibodies. The displayed bi-specific antibodies
may be affinity selected for binding to the target protein to

obtain high affinity binding phages. Uisng the same strategy,
bi-specific antibodies may be obtained that bind to two
epitopes that are expressed on different molecules.

5   It is also contemplated by the present invention that
individual antibodies or peptides, and the genes or
oligonucleotides encoding these antibodies or peptides, can
be isolated from the phage display library after affinity
enrichment. These genes and oligonucleotides can be used for
10   subcloning into expression vectors for production of
antibodies and derivatives of the oligonucleotides can be
used for nucelotide se quencing and the information used to
produce chemically synthesized peptides or peptides produced
by recombinant DNA methods such as production in prokaryotic
15   or eukaryotic expression systems.
In an alternative embodiment the present invention provides a
method for identifying a peptide capable of specific binding
to a fixated biological target, comprising displaying the
peptide on the surface of a replicable display package and
20   contacting said specific binding peptide with the fixated
target to allow for binding.

Thus the present invention provides a method that facilitates
the selection of antibodies that are suitable for
25   immmunohistochemical and immunofluorescent procedures with
tissue sections that have been embedded and fixed with a
variety of procedures. In addition, the procedure facilitates
the isolation of antibody specificities that bind to
particular anatomical regions of a tissue section, for
30   example a region infiltrated by tumor cells by using a
subtraction procedure and/or micromanipulation of individual
cells or small groups of cells. The method employs large
libraries of antibodies or peptides expressed on the surface
of a replicable display package. The display libraries may be
35   constructed by a variety of methods. In an exemplary
embodiment the display package is a phage particle which

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comprises an antibody-coat protein fusion; the nucleotide
sequences used for construction of the antibodies are
partially synthesized and assembled in vitro. These libraries
contain many novel specificities, including antibodies
5   against self antigens.

     In the basic approach, phages expressing antibody
     specificities of interest are selected from this library in
     again a four step procedure 1) binding of phages to the
10   embedded and fixed tissue sections, 2) removal of non-bound
     phages by washing 3) elution of bound phages and 4) infection
     and propagation of eluted phages in E. Coli bacteria. To
     obtain specific phage antibodies that bind to a particular
     tissue or cell type, the method may be extended by absorbing
15   eluted phage antibodies to remove unwanted specificities.
     This may be achieved by incubating eluted phage antibodies
     with sections of other tissues or with sections of the same
     tissue not containing the target cell of interest and
     subsequent propagation of unbound phages. In addition, to
20   obtain phage antibodies specific for a particular cell type,
     for example defined by anatomical localization, individual or
     groups of cells or pieces of tissue and attached phages may
     be scraped from the tissue section by a micromanipulator.

25   The present invention makes available a powerful directed
     approach for isolating specific antibodies or peptides by
     antibody or peptide display technologies in combination with
     solid-phase-dependent peptide synthesis.

30   The present invention further makes available a powerful
     directed approach for isolating specific antibodies or
     peptides by antibody or peptide display technologies in
     combination with selection procedures on tissue sections or
     whole tissues.

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In one exemplary embodiment of the present invention, the
display package is a phage particle which comprises an
antibody fusion coat protein that includes the amino acid
sequence of an antibody variable region. A library of
5   replicable phage vectors, especially phagemids encoding a
library of antibody fusion coat proteins is generated and
used to infect suitable host cells. The antibody variable
genes used to construct the phage library may be entirely
derived from the lymphocytes of individuals or (partly)
10   assembled in vitro as in semi-synthetic phage antibody
display libraries. An important determinant of the
composition and diversity of these in vitro immune systems is
the source of antibody genes used as building blocks to
construct the library. Libraries may be assembled from the V
15   regions expressed by the B lymphocytes of an individual known
to have mounted a particular immune response as a result of
immunization or exposure to an infectious agent. The B cell
repertoire in the lymphoid organs of these individuals is
enriched for antigen-specific B cells and plasma cells that
20   have undergone clonal expansion and antigen affinity
maturation, thus increasing the likelihood of selecting high
affinity antibody fragments from the eventual "immunized"
phage library. Libraries may be constructed from the V
regions expressed by the B cells of "non-immunized"
25   individual in an attempt to recruit all the diversity
generated by the natural immune system. Importantly, it
should be noted that the B lymphocytes of naive, immunized or
infected individuals have been through positive and negative
selection forces in primary and secondary lymphoid organs.
30   The net effect of these processes is a B cell repertoire that
is less diverse than potentially attainable, based on
rearrangement of immunoglobulin gene segments, N region
insertions and somatic hypermutation. This in vivo antibody
repertoire selection is mirrored in the eventual "naive"
35   phage library. An alternative approach to create diverse
libraries exploits the use of large collections of cloned V

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genes to which randomized CDR3 and JH regions are fused *in vitro* by PCR. The diversity of these "semi-synthetic" libraries is not constrained by the forces of selec tion acting in the natural immune system. It may be anticipated

5   that such libraries contain specificities not present in the actual antibody repertoires of humans. In the exemplary embodiment a synthetic library is used because it is expected to contain the novel anti-peptide specificities to be selected.

10

Phage particles formed from the chimaeric protein can be separated by affinity selection based on the ability of the phage-associated antibody to bind to the target peptide synthesized on rods of a pepscan block. The rods on the

15   pepscan block are decorated with individual peptides, the amino acid sequence of which is determined by the amino acid sequence of the target protein. After one or multiple rounds of selection on peptides synthesized on the pepscan block, the phages are selected for binding to the target protein in

20   its native or denatured configuration. For example, the target protein may be a purified recombinant protein expressed in eukaryotic or prokaryotic cells, or a recombinant protein expressed on the cell surface of a prokaryotic or eukaryotic cell. Phages binding to the target

25   protein are eluted, propagated and eventually analyzed for binding.

After one or multipe rounds of affinity selection on peptides, a subtractive or absorption step may be included to

30   obtain phages that discriminate between two closely related peptides. For example, phages that discrimintate between the closely related peptide repre sented by the amino acid sequences DLVYKDPARPKI and DLVYKDPYRPKI may be obtained by affinity selection on the amino acid sequence DLVYKDPARPKI,

35   followed by absorption of phages binding to the amino acid sequence DLVYKDPYRPKI. In the latter step, non-discriminative

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phages recognizing both peptides are removed and not used for
propagation. Antibodies produced by this method can for
example be used to distinguish various naturally occuring
isoforms of a protein or for immunochemical assays for
5   detecting cell transformations arising due to mutation of an
oncogene or an anti-oncogene.

The terms absorption and subtraction refer to the removal of
phage antibodies with unwanted specificities from a
10 collection of phages obtained after or prior to affinity
selection. The absorption step itself is an affinity
selection step on a target expected not to bear the epitopes
of interest. The target used for absorption/selection may be
tissue sections, cell lines recombinant proteins, peptides
15 etc.

Examples

The invention now being generally described, it will be more
20 readily understood by reference to the following examples
which are included merely for the illustration of certain
aspects and embodiments of the present invention and are not
intended to limit the invention.

25 I. Materials and Methods

Except were indicated otherwise, recombinant DNA methods and
microbiological techniques were carried out using standard
procedures such as described for example by Sambrook, J. et
30 al. Molecular Cloning: A Laboratory Manual, Cold Spring
harbor Laboratory Press, Cold Spring Harbor, NY (1989) and
Ausubel, F.M. et al. Current Protocols in Molecular Biology,
John Wiley and Sons, Inc. (1995). Specific protocols for
propagation and selection of phage antibodies are described
35 in de Kruif, J., et al. Methods in Molecular Biology,
(1996?).

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Construction of a synthetic phage antibody display library

The synthetic antibody library was constructed essentially as
5   described (de Kruif et al., J. Mol. Biol. 248, 97 (1995)).
Briefly, degenerate oligonucleotides were used to add
synthetic CDR3 regions to a collection of 49 previously
cloned germline VH genes. Subsequently, these _in vitro_
'rearranged' VH genes were cloned into a collection of pHEN1
10   phagemid-derived vectors containing 7 different light chain V
regions, fused in frame to the gene encoding the phage minor
capsid protein geneIII. Introduction of these constructs into
bacteria results, in the presence of helper phage, in the
expression of scFv antibody fragments as geneIII fusion
15   proteins on the surface of bacteriophage.

Preparation of tissue sections

Fixation, freezing, embedding and sectioning of tissues were
20   carried out using standard procedures such as for example
described in Zeller, R in Current Protocols in Molecular
Biology, pp 14.1.1-14.1.8, Ausubel, F.M. et al. Eds, Green
Publishing and Wiley Interscience, New York.
Immunohistochemical and immunofluorescent procedures were
25   carried out using standard protoclos such as for example
described in Current Protocols in Immunology, pp 5.8.1-5.8.8,
Coligan, J.E., et al. Eds. Wiley Interscience, New York.

Example I: selection using pepscan

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Pepscan synthesis of CD64 peptides

Pepscan synthesis was carried out essentially as described
(Geysen, H.M., et al, Proc. Natl. Acad. Sci. USA, 81, 3998
35   (1984)). In the exemplary embodiment, the extracellular
domain of the CD64 molecule was synthesized on 25

polyethylene rods as adjacent, non-overlapping sets of 12-mer
peptides, according to the scheme in Table I.
Peptide-specific phages were isolated by affinity binding to
the CD64 peptide-covered rods. The rods were blocked by

5   incubation for two hours at room tempertaure by placing the
pepscan block for two hours at roomtemperature in wells of a
96-well microtiter plate containing 300 microliter 1x
blocking buffer per well (2x blocking buffer: 10%
ovalbumin/10% horse serum/1% Tween 80 in PBS). The phages

10   were were mixed 1:1 (v/v) with 2x blocking buffer and
incubated for 30 minutes at room temperature. 250 microliter
of the blocked phage solution containing approximately 10^{12}
phages were added per well of a 96 well plate and the 25 rods
of the pepscan block were submerged in individual wells and

15   incubated for 3 hours at roomtempera ture. Non-specific phage
antibodies were then removed by washing the rods 10 times in
PBS/0.5% Tween 80 and one final time in PBS. Phage antibodies
bound to the rods were then eluted by adding 300 microliter
of 0.1M diethylamine buffer to each well of a microtiter

20   plate and incubation for 5 minutes at roomtemperature. After
collection of the 300 microliter, the elution step was
repeated. To 600 microliter of eluted phages, 300 microliter
of 1M Tris buffer pH 7.4 was added for neutralization.The 900
microliter eluted phage suspension was added to 4 ml 2TY

25   containing 5% glucose and 10 microgram/ml tetracycline.
Finally, 5 ml XL-1 blue cells (OD600 of 0.5) were added and
the mixture incubated for 30 minutes at 37°C. The cells were
centrifuged for 30 minutes at 3000 rpm, the supernatant
removed by aspiration and the cells resuspended in 500

30   microliter of the remaining medium on two TYE plates containg
5% glucose, 100 microgram/ml ampicillin, 10 microgram/ml
tetracycline and 5% glucose and the bacteria grown overnight
at 37° C. The colonies were scraped from the bacterial plates
and used to rescue phage antibody particles as described (de

35   Kruif, J., et al., J. Mol. Biol. 248, 97 (1995)). From the
resulting library enriched for peptide-binding phage

antibodies, 10^{12} phage antibodies in blocking buffer were
incubated with the same peptide-covered rods and subjected to
subsequent rounds of selection using the same procedure.

5   Cleaning of pepscan blocks

Blocks used for ELISA or phage selections were cleaned by
submerging the pep scan blocks in disruptbuffer
(phosphatebuffer/1%SDS/0.1%beta-mercapto-ethanol, pH 7.2) and
10   placing the block in a ultrasonic waterbath at 70°C for 1
hour.

Analysis of selected phages in ELISA

15   After selection, preparations of monoclonal or polyclonal
scFv antibodies were analyzed for the presence of peptide
binding in ELISA using the same pepscan blocks used for
selections. ScFv preparations instead of phage preparations
were used because the latter generated a high background in
20   ELISA. All incubations are carried out in 96-well microtiter
plates. The cleaned rods were soaked in 300 microliter PBS
for 30 minutes at roomtemperature and pre-coated by
incubating each pin for 3 hours at roomtemperature with 150
microliter of blocking buffer (5% ovalbumin/5% horse serum/1%
25   Tween 80, filtered over Whatman 41 paper). The rods were
rinsed twice with PBS/0.05% Tween 80 and incubated for 1 hour
at roomtemperature with 150 microliter scFv-containing
periplasmic preparations prepared as described in de Kruif et
al, J. Biol. Chem. 271, 7630 (1996)) mixed with 150
30   microliter blocking buffer. The rods were washed twice with
PBS/0.05% Tween 80 and incubated for 1 hour with 300
microliter culture supernatant of a hybridoma secreting the
anti-Myc tag antibody 9E10 (de Kruif, J., et al, J. Biol.
Chem. 271, 7630 (1996)). The rods were washed twice with
35   PBS/0.05% Tween 80 and incubated for 1 hour with 300
microliter of rabbit anti-mouse immunoglobulins conjugated to

18

peroxidase, diluted 1:500 in PBS/0.05% Tween 80/1% BSA.
Finally, the rods were washed thrice with PBS/0.05% Tween 80
and incubated with ABTS substrate. A representative result of
an anti-peptide scFv antibody, affinity-selected after three

5   rounds for binding to peptide # 21 on the CD64 block is shown
in figure 2. This scFv antibody specifically bound to peptide
#21 used as target for selection but not to other
peptide-covered rods in the CD64 pepscan block (figure 2).

10   <u>Selection and analysis of pepscan pre-selected phage
antibodies by cell sorting.</u>

Phage populations enriched for binding to peptides on the
pepscan block may in the second or subsequent rounds be

15   selected for binding to prokaryotic or eukaryotic cells
expressing the target protein employing flow cytometry and
cell sorting. The procedure for phage selection on intact
cells using this procedure has been descibed in detail (de
Kruif, J., et al. Proc. Natl. Acad. Sci. USA, 92,3938).

20   Approximately 10^{13} phage antibody particles are blocked for
15 minutes in 4 ml 4% milkpowder in PBS (MPBS). $5 * 10^6$
target cells were added to the blocked phages and the mixture
was slowly rotated overnight at 4°C. The following day, cells
were washed twice in 50 ml ice-cold PBS/1% BSA. The pelleted

25   cells were resuspended in 2 ml of ice-cold PBS/1% BSA for
cell sorting or stained with fluorochrome-labeled monoclonal
antibo dies for sorting of a subpopulation of cells. To that
end, 25 microliter of fluorochrome-labeled antibodies is
added to the pelleted cells and after a 20 minute incubation

30   on ice, cells were washed once with 1% BSA/PBS and
resuspended in 2 ml ice-cold PBS/1% BSA. Cell sorting was
performed on a FACSvantage.

The procedure for flow cytometric analysis of selected phage

35   antibodies has been described in detail elsewhere (de Kruif,
J., et al. Proc. Natl. Acad. Sci. USA, 92,3938). For staining

of cells, 100 µl monoclonal phage antibody containing
approximately 10^{11} particles was blocked by adding 50 µl 4%
MPBS for 15 minutes at roomtemperature. $5*10^5$ leucocytes in
50 µl PBS/1% BSA were added and incubated on ice for 1 hour.

5   The cells were washed twice in ice-cold PBS/1% BSA. To detect
cell-bound phages, the cells were incubated in 10 µl of 1/200
diluted sheep anti-M13 polyclonal antibody (Pharmacia,
Uppsala. Sweden), washed twice and incubated in 10 µl of 20
µg/ml PE-labeled donkey anti-sheep polyclonal antibody

10   (Jackson Immu noresearch, West Grove, PA), each for 20
minutes on ice.

Selection of pepscan pre-selected phage antibodies on whole
tissues or tissue sections.

15

Tissue sections from target organs are prepared and fixed by
standard methods. For example, tissues may be submerged in
OCT embedding medium and gently frozen by dipping in liquid
nitrogen for 1 minute. Using a cryostat, 10 micrometer frozen

20   sections are prepared and collected on microscope slides. The
sections are dried for 10 minutes at roomtemperature and
fixed in 100% acetone for 5 seconds, followed by drying at
roomtemperature. Before incubation with the phage library,
the tissue sections are sealed with wax to prevent spreading

25   of fluid and blocked by incubation with MPBS for 30 minutes
at roomtemperature. After the blocking step, a total of 10^{12}
phages from a phage antibody library pre-selected for
binding to peptides is blocked with MPBS in a volume of 100
microliter and added to the droplet of MPBS on the slide. The

30   tissue sections are incubated overnight in a moist chamber at
40°C to facilitate binding of phage antibodies.

The sections are rinsed with a total of 2 liter PBS/0.5%
Tween 20 for 2 hours at roomtemperature with ten 200 ml

35   changes of PBS/0.5% Tween 20. After rinsing, the phage
antibodies are eluted by placing 300 microliter 76 mM citric

20

acid buffer (pH 2.5) on the slide and incubation for 5
minutes at roomtemperature. Add 150 microliter of 1 M
Tris-HCL buffer are added to the slide for neutralization and
the suspensi on is brought in 3 ml 2 TY medium in a 15 ml
5   tube. 3 ml of XL-1 blue bacteria is added to the tube and
infection and production of phages are allowed to proceed as
described in the standard protocol. Afer 2-3 rounds of
selection, monoclonal phage antibodies are prepared for
analysis.
10

Analysis of selected phage antibodies by immunohistochemistry

Binding of monoclonal phage antibodies to tissues was
determined by immunohistological screening. To this end, 5 μm
15 frozen tissue sections were collected on gelatin coated
microscope slides. Sections were then incubated with 50
microliter phage solutions containing 10^{10} phage particles in
a moist chamber for 30 min. Non-adherent phages were removed
by vigorously rinsing the slides in M-PBS-Tw. Bound MoPhabs
20 were visualized by incubation of the sections with a 1:50
diluted sheep anti-M13 peroxidase conjugate, followed by
incubation with the substrate diamino benzidene.

Construction and selection of bi-specific antibodies from
25 collections of phages antibodies affinity selected on pepscan
blocks.

Monoclonal phage antibodies selected for binding to the CD64
peptides may be pooled and recloned in display vectors
30 permitting the display of bi-specific antibodies such as for
example vectors for diabodies (Holliger, P. et al., Proc.
Nat. Acad. Sci. USA, 90, 6444 (1993) or leucine zippered
bi-specific antibodies (de Kruif, J., et al. J. Biol. Chem.
271, 7630 (1996).
35

21

In the exemplary embodiment, a phagemid vector was
constructed that facilitates the in vivo bacterial
expression and assembly of bispecific (scFv)2 fragments as
secreted molecules or g3p fusion proteins on the surface of
5   bacteriophages. The Fos and Jun scFv leucine zipper
constructs we described (de Kruif, J., et al. J. Biol. Chem.
271, 7630 (1996); figure 3, 1 and 2) are PCR amplified using
primers M13R and PDIM3 (5'-TTT GCA TTC AAG CTT TTA TTA GCC
CGC ATA GTC AGG AAC ATC GTA TGG GTA TGC GGC AGC GCA ACC ACC).
10   Primer PDIM3 replaces the Myc tag with a haemagglutinin (HA)
tag and adds a stopcodon and a HindIII site to these
fragments. PCR products are digested with HindIII and cloned
into vector pHEN1 (Hoogenboom, H.R., et al., Nucleic Acid
Res. 19, 4133 (1991)) containing a scFv gene fused to the
15   complementary leucine zipper and a Myc tag. In the resulting
constructs both scFv-zipper fragments are encoded by a single
transcript. Two ribosome binding sites allow the proteins to
be translated individually, the first scFv fragment fused to
a Fos zipper domain and an HA tag, the second scFv to a Jun
20   zipper and a Myc tag (Figure 3, 3). To determine the effect
of the position of the scFv-zippers relative to the g3p
protein, an additional construct was made in which the
scFv-zipper fragments were reversed (Figure 3, 4). In
suppressor E . coli strains, an amber codon inserted between
25   the Myc tag and gene 3 permits the production of phage
particles displaying a scFv fragment fused via a zipper
region to gene 3. The second scFv will associate with the
phage in the periplasmic space by heterodimeri zation of the
Fos and Jun leucine zippers, thus creating a phage expressing
30   a bispecific antibody on its surface. In non-suppressor
strains, bispecific (scFv)2 fragments linked by a Fos Jun
leucine zipper will be assembled in the periplasmic space.

Expression of (scFv)2 fragments in non-suppressor bacterial
35   strain SF110-FÂ was monitored by SDS-PAGE followed by

22

blotting to nitrocellulose and detection with Myc tag- and HA tag-specific monoclonal antibodies (MoAbs) (Figure 4, lower panel). In reducing SDS-PAGE, constructs 3 and 4 show expression of HA and Myc tagged proteins of the predicted

5   size. The expression level of Myc tagged scFv fragments obtained with constructs 3 and 4 is lower when compared to that obtained with constructs 1 and 2, perhaps as a result of the simultanuous expression of a second scFv in bacteria harboring the former constructs. In non-reducing SDS-PAGE,

10 only proteins approximately twice the size of a scFv-zipper are visible in all lanes (Figure 4, upper panel). The anti HA-MoAb detects multiple bands, whereas the anti Myc-MoAb detects only one. One of the fragments visualized with the anti-HA MoAb migrates at the same position as the fragments

15 visualized with the anti-Myc MoAb. We hypothesize that this band represents the bispecific antibody and that the other HA-tagged proteins are homodimers resulting from a higher expression level of the first scFv-zipper in these constructs.

20
    Functionality of the (scFv)$_2$ fragments was analysed in an ELISA (Figure 5). Using both the Myc tag- and HA tag-specific Moabs for detection, specific binding of the (scFv)$_2$ fragments to both target antigens could be demonstrated. In a

25 sandwich ELISA we show the (scFv)$_2$ fragments are truly bispecific in linking two different antigens.
    Recombinant phage antibody particles were produced using constructs 1-4 and analysed in an ELISA (Figure 5). Phages from constructs 2 and 3, containing the Jun-zipper fused

30 directly to gene 3 show some non-specific binding to irrelevant antigens. Phages produced using constructs 1 and 4, containing the Fos-zipper fused to gene 3 recognize their target antigens only.

35 To test the feasability of selecting bispecific phage antibodies from a phage repertoire, bispecific phage

23

particles from construct 4 were spiked in a 1:1000 ratio in
an antibody phage display library and subjected to one round
of selection in immunotubes coated with either IgG or DNP.
The frequency of bispecific phages after selection was
5   estimated by PCR on individual colonies using primers M13R
and FOSCON (5'-CGC CAG GAT GAA CTC C, situated in the Fos
zipper). Frequencies of bispecific phages after selection
were 0/32 (spiked library), 9/32 (selected on IgG) and 6/32
(selected on DNP). Calculated enrichment factors (±250x) are
10   comparable to those obtained using monospecific phage
antibodies.

This vector permits the expression of bispecific antibodies
that can be functionally expressed on phages using Fos and
15   Jun leucine zippers. It is therefore possible to construct
libraries of phages expressing two different scFv fragments
and select the desired combinations from these. Furthermore,
selected bispecific (scFv)2 fragments can be produced in
non-suppressor strains and purified using anti-Myc followed
20   by anti-HA affinity columns.

For the selection of bi-specific antibodies from phage
antibody collections pre-selected for binding to peptides on
pepscan blocks, care is taken to restrict the number of
25   selection rounds (usually 2-3) on the rod-bound peptides to
ensure enrichment for binders but to prevent loss of
diversity by overselection. The V gene pools encoding the
phage antibodies enriched for binding to the peptides are
recloned into the leucine-zipper constructs and bi-specific
30   phage antibodies binding to the target protein are selected
by any of the afore-described methods. In addition, it may be
envisaged that higher affinity bi-specific antibodies are
directly isolated by performing phage selection with the aid
of surface palsmon resonance (Jonsson, U., et al.
35   Biotechniques, 11: 620 (1991).

24

Example 2: selection on sections
Preparation of tissue sections

Tissue sections from target organs are prepared and fixed by

5   standard methods. For example, tissues may be embedded in an
    embedding compound such as OCT and gently frozen by dipping
    in liquid nitrogen for 1 minute. Using a cryostat, 10
    micrometer frozen sections are prepared and collected on
    microscope slides. The sections are dried for 10 minutes at

10 roomtemperature and fixed in 100% acetone for 5 seconds,
    followed by drying at roomtemperature.

    Alternatively, tissues may be fixed by incubation for several
    hours in 4% freshly prepared paraformaldehyde solution,

15 dehydrated by serial passage through increasing
    concentrations of ethanol and embedding in paraffin wax.
    Parafin blocks are cut into 6-8 micrometer sections.
    Instead of tissue sections, freshly isolated or cultured
    cells may be used and subjected to the same procedures.

20
    Affinity selection of phages on tissue sections

    The frozen tissue sections are briefly dried at room
    temperature. The parraffin-embedded tissue sections are

25 placed at 60°C for 1 hour and deparaffinized by immersing in
    three changes of fresh xylene, 4 minutes each, followed by
    two changes of 100% ethanol, 2 minutes each. Tissue sections
    are rehydrated in solutions of decreasing concentrations of
    ethanol and finally water, and washed with PBS.

30
    Before incubation with the phage library, the tissue sections
    are sealed with wax to prevent spreading of fluid over the
    object glass, and blocked by incubation with 1% non-fat
    milkpowder in phosphate buffered saline (MPBS) for 30 minutes

35 at roomtemperature. After the blocking step, a total of 10^{13}
    phages from the synthetic phage antibody display library is

blocked with MPBS in a volume of 300 microliter and added to
the droplet of MPBS on the slide. The tissue sections are
incubated over night in a moist chamber at 40°C to facilitate
binding of phage antibodies.

The sections are rinsed with a total of 2 liter PBS/0.5%
Tween 20 for 2 hours at roomtemperature with 10 200 ml
changes of PBS/0.5% Tween 20. After rinsing, the phage
antibodies are eluted by placing 300 microliter 76 mM citric
acid buffer (pH 2.5) on the slide and incubation for 5
minutes at roomtemperature. 150 microliter of 1 M Tris-HCL
buffer is added to the slide for neutrlaization and the
suspension is brought in 3 ml 2 TY medium in a 15 ml tube. 3
ml of XL-1 blue bacteria are added to the tube and infection
and production of phages are allowed to proceed as described
in the standard protocol. Afer 2-3 rounds of selection,
monoclonal phage antibodies are prepared for analysis by
immunohistochemistry.

Analysis of selected phage antibodies by immunohistochemistry

Binding of monoclonal phage antibodies to tissues was
determined by immunohistological screening. To this end, 5 μm
frozen tissue sections were collected on gelatin coated
microscope slides. Sections were then incubated with 50
microliter phage solutions containing approximately 1010
phage particles in a moist chamber for 30 min. Non-adherent
phages were removed by vigorously rinsing the slides in
MPBS-Tw. Bound phage antibodies were visualized by incubation
of the sections with a 1:50 diluted sheep anti-M13 peroxidase
conjugate, followed by incubation with diaminobenzidene
substrate.

Absorption of selected phages antibodies for unwanted
specificities

A collection of phage antibodies selected for binding to a
5   tissue section may be absorbed for unwanted phage antibody
specificities. After elution and neutralization of bound
phage antibodies, a 4% (w/v) MPBS solution is added to the
eluate to reach a 1% concentration of non-fat milk. The phage
solution is subsequently incu bated with a tissue sections of
10   for example another tissue or a different anatomical region
of the same tissue. Non-binding phages are removed and
propagated directly or incubated with another tissue secion
for another round of absorption. This absorption procedure
may be repeated multiple times before the eventual
15   non-binding phages are propagated in bacteria.

Isolation of specific phage antibodies from tissue sections
using a micromanipulator.
20
Individual cells or small regions of tissue and binding
phages may be scraped from tissue sections using a
micromanipulator or similar device, essentially as described
(Kupper, R., et al. EMBO-J, 12 4955 (1993)). This procedure
25   allows the isolation of phages that bind to particular
anatomical structure or individual cells in a tissue section.
For example, this procedure allows the isolation of
endothelial of bloodves sels penetrating a tumor mass or
allow the isolation of individual tumor cells. Once scraped
30   from the tissue secion, the bound phages are eluted and
propagated as described in the basic protocol.

Legends to the figures

Figure 1.
General principle of affinity selection of genetic display
5   packages by binding to peptides synthesized on the rods of a
pepscan block.

Figure 2.
ELISA results of a phage antibody affinity selected for
10 binding to peptide #21 on the CD64 pepscan block. No binding
above background to other peptides on the block was observed.

Figure 3.
Starting material (1 and 2) and bispecific constructs (3 and
15 4). LacZ promoters (triangles), ribosome binding sites
(circles) pelB leaders (P), Fos-zippers (F), Jun-zippers (J),
Myc tag (M), HA tag (H) and stopcodons (arrows) are
indicated. Closed boxes and hatched boxes represent different
scFv specificities.
20

Figure 4.
SDS-PAGE / Western blotting analysis. Periplasmic
preparations from expressed constructs 1-4 were run under
non-reducing (top panel) and reducing (bottom panel)
25 conditions. Proteins were visualized using an HA-tag specific
MoAb (left lanes) and a Myc-tag specific MoAb (right lanes)
followed by a peroxidase- conjugated antibody.

Figure 5.
30 ELISA analysis using construct 1-4 derived antibody
fragments. In standard ELISA's, $(scFv)_2$ fragments were
allowed to bind to antigen coated wells and detected using
anti-Myc and anti-HA MoAbs followed by a
peroxidase-conjugated antibody. Sand wich ELISA's using
35 bispecific $(scFv)_2$ fragments were performed as described (de
Kruif, J et al. J. Biol. Chem. 271, 7630). In phage ELISA,

28

phage antibodies were allowed to bind to antigen coated wells
and developed using a peroxidase conjugated anti-M13
antibody. Numbers represent absorbance values. Values three
times higher than the background binding to milk-blocked
wells are considered to be positive (gray boxes).

29

pin #	amino acid sequence	pin #	amino acid sequence
1	MWFLTTLLLWVP	14	KTNISHNGTYHC
2	VDGQVDTTKAVI	15	SGMGKHRYTSAG
3	SLQPPWVSVFQE	16	ISVTVKELFPAP
4	ETVTLHCEVLHL	17	VLNASVTSPLLE
5	PGSSSTQWFLNG	18	GNLVTLSCETKL
6	TATQTSTPSYRI	19	LLQRPGLQLYFS
7	TSASVNDSGEYR	20	FYMGSKTLRGRN
8	CQRGLSGRSDPI	21	TSSEYQILTARR
9	QLEIHRGWLᴊLQ	22	EDSGLYQCEAAT
10	VSSRVFTEGEPL	23	EDGNVLKRSPEL
11	ALRCHAWKDKLV	24	ELQVLGLQLPTP
12	YNVLYYRNGKAF	25	VWFHVLFYLAVG
13	KFFHWNSNLTIL		

Table 1. Sets of oligopeptides representing the extracellular domain of CD64.

30

CLAIMS

1.   A method for identifying a peptide capable of specific binding to a proteinaceous target, comprising displaying the peptide on the surface of a replicable display package, synthesizing oligopeptides derived from the proteinaceous
5   target on a solid phase and contacting the specific binding peptide with the oligopeptide to allow for binding.

2.   A method for identifying a peptide capable of specific binding to a fixated biological target, comprising displaying the peptide on the surface of a replicable display package
10   and contacting said specific binding peptide with the fixated target to allow for binding.

3.   A method for distinguishing between peptides capable of specific binding to a proteinaceous antigen and peptides not having that capability comprising displaying candidate
15   peptides on the surface of a replicable display package, synthesizing oligopeptides derived from the proteinaceous antigen on a solid phase and contacting the candidate peptides with the oligopeptides to allow for binding and washing the solid phase to remove the display packages not
20   specifically bound.

4.   A method for distinguishing between peptides capable of specific binding to a fixated biological target and peptides not having that capability comprising displaying candidate peptides on the surface of a replicable display package,
25   contacting said specific binding peptide with the fixated target to allow for binding and washing the fixated biological target to remove the display packages not specifically bound.

5.   A method according to anyone of the afore going claims,
30   whereby the replicable display package is a phage particle.

6.   A method according to anyone of the afore going claims, whereby the replicable display package is a bacterium, a yeast or a spore of a microorganism.

7.   A method according to claim 5, whereby the specific
binding peptide is diplayed on the surface of the phage by
insertion of its encoding sequence in a gene encoding a
surface protein of said phage.

5   8.   A method according to anyone of the aforegoing claims,
whereby the displayed peptide is an immunoglobulin heavy
chain, an immunoglobulin light chain, a heavy-light chain
pair, a VH, a VL, a Fab, a Fv, an scFv or a
di-sulfide-bridged Fv.

10   9.   A method according to anyone of the afore going claims
whereby the specific binding peptide is a single chain
antibody fragment, preferably an ScFv.

10.   A method according to anyone of the aforegoing claims,
further comprising a step whereby the displayed peptides are
15   contacted with a sample not containing the target of
interest.

11.   A method for screening a library of replicable display
packages for peptides capable of specific binding to a
prteinaceous target or a fixated biological target comprising
20   subjecting the peptides in the library to a method according
to any one of the aforegoing claims.

12.   A method according to claim 11 wherein said library is
a phage display library.

protein

peptides

pepscan-block

phage-antibodies
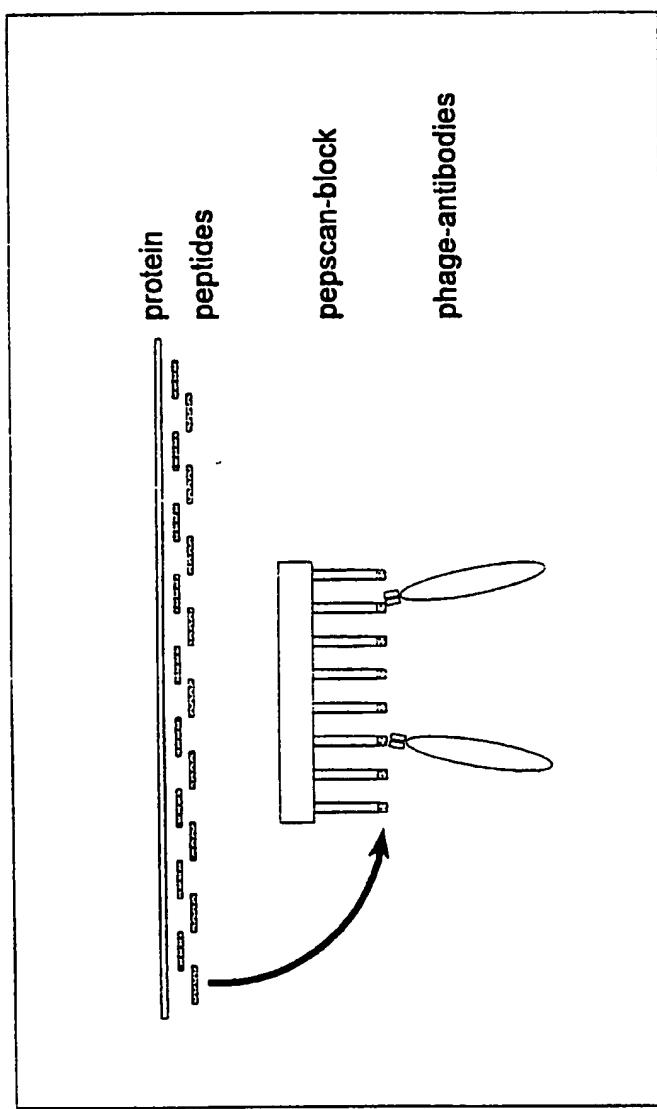
Figure 1

CD64 pepscan using scFv clone F2

A 405 nm
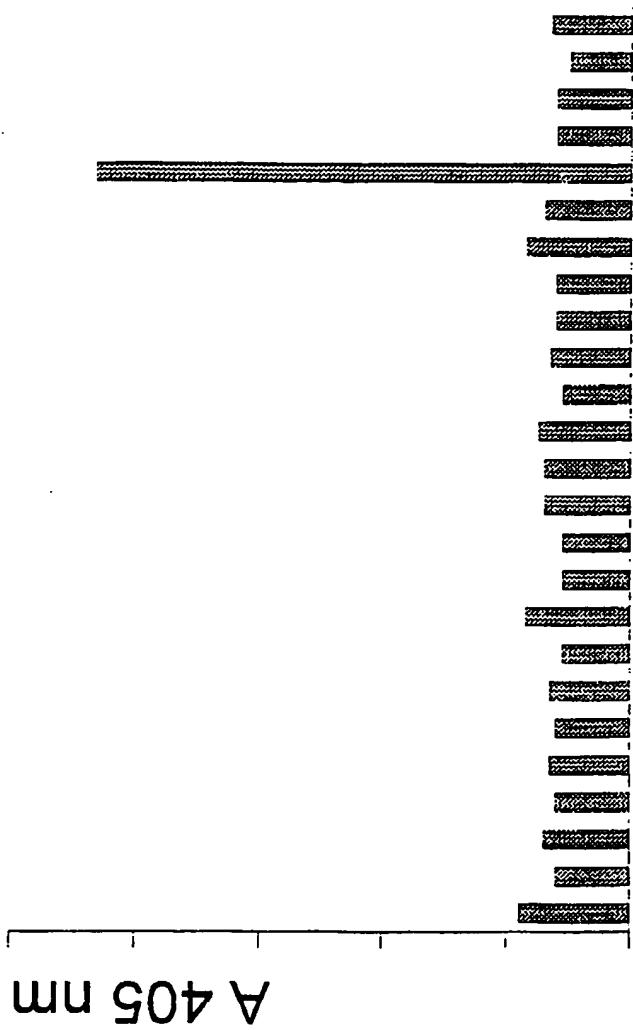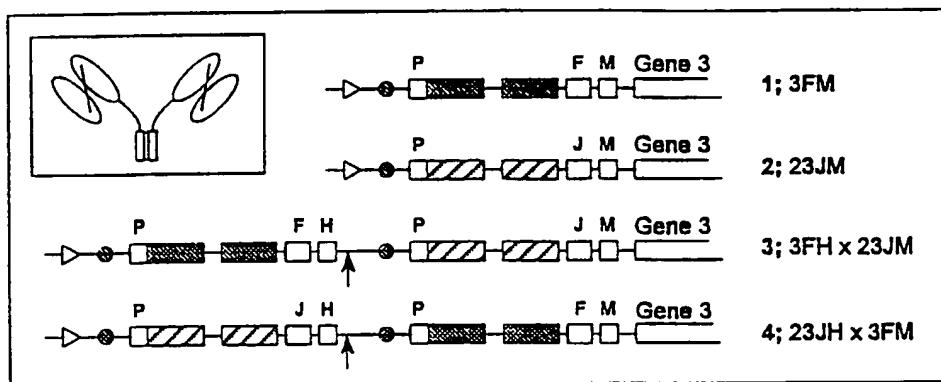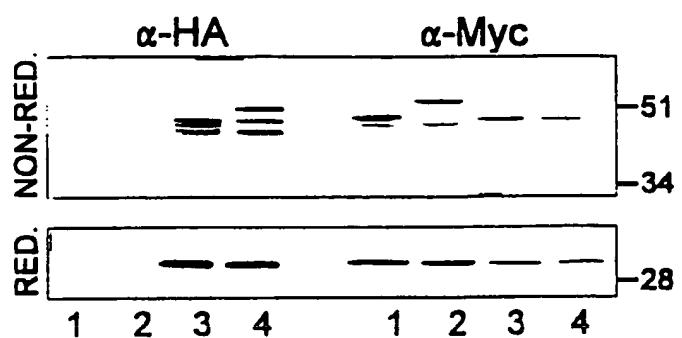
Figure 2

Figure 3

Figure 4

	1	2	3	4	
IgG	2.11	0.09	0.61	0.26	
DNP	0.07	2.63	1.74	1.22	$(scFv)_2$; α-Myc
Milk	0.06	0.08	0.06	0.06	
IgG	0.07	0.07	1.62	0.56	
DNP	0.07	0.06	1.95	2.26	$(scFv)_2$; α-HA
Milk	0.06	0.06	0.06	0.06	
IgG + DNP	0.06	0.08	0.79	0.51	$(scFv)_2$; sandwich
IgG	1.56	0.55	0.96	1.05	
DNP	0.09	1.81	1.62	1.19	biphab; α-M13
Milk	0.06	0.62	0.35	0.09	

Figure 5

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6   G01N33/554   G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6   G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 92 15677 A (PROTEIN ENGINEERING CORP) 17 September 1992 see the whole document ---	1,3,5-7, 11,12
X	DE KRUIF ET AL.: "Selection and application of human single chain Fv antibody ...." JOURNAL OF MOLECULAR BIOLOGY, vol. 248, no. 1, 21 April 1995, LONDON, pages 97-105, XP000196590 cited in the application see the whole document	1,3,5, 8-12
Y	---	2,4,6,7
Y	WO 92 08738 A (ABBOTT LAB) 29 May 1992 see page 8, line 21 - line 23 --- -/--	2,4

[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

° Special categories of cited documents :

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
2 December 1997	19. 12. 97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Ceder, O

2

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 95 15982 A (GENZYME CORP) 15 June 1995 see claims 1-5,65 ---	6
Y	WO 95 31723 A (FRYKBERG LARS ;JACOBSSON KARIN (SE)) 23 November 1995 see abstract ---	7
A	GEYSEN ET AL: "USE OF PEPTIDE SYNTHESIS TO PROBE VIRAL ANTIGENS FOR EPITOPES TO A RESOLUTION OF A SINGLE AMINO ACID" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 81, July 1984, WASHINGTON DC, pages 3998-4002, XP000609021 cited in the application see abstract -----	1,3

2

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9215677 A	17-09-92	US 5223409 A	29-06-93
		JP 7501923 T	02-03-95
		AU 1545692 A	06-10-92
		AU 1578792 A	06-10-92
		AU 1581692 A	06-10-92
		AU 8740491 A	28-04-92
		CA 2105300 A	02-09-92
		CA 2105303 A	02-09-92
		CA 2105304 A	02-09-92
		EP 0575485 A	29-12-93
		EP 0573603 A	15-12-93
		EP 0573611 A	15-12-93
		JP 6510522 T	24-11-94
		JP 7501203 T	09-02-95
		US 5403484 A	04-04-95
		US 5571698 A	05-11-96
		WO 9206191 A	16-04-92
		WO 9215605 A	17-09-92
		WO 9215679 A	17-09-92
		US 5663143 A	02-09-97
WO 9208738 A	29-05-92	AU 662503 B	07-09-95
		AU 9077791 A	11-06-92
		CA 2095735 A	08-05-92
		EP 0556320 A	25-08-93
		JP 6502076 T	10-03-94
WO 9515982 A	15-06-95	AU 1432195 A	27-06-95
		EP 0733070 A	25-09-96
		JP 9506262 T	24-06-97
		CA 2175482 A	15-06-95
WO 9531723 A	23-11-95	AU 2541595 A	05-12-95
		EP 0760100 A	05-03-97